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## Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Research Report.....	4-9
Key Research Accomplishments.....	9
References.....	10-11

## A. INTRODUCTION

**A-1. Identification of genes differentially expressed in breast cancer versus normal breast.** We undertook a search, using the differential cDNA sequencing approach as we previously described (1-3), for isolation of differentially expressed genes in the cDNA libraries from normal breast and breast carcinoma. Of many putative differentially expressed genes, a breast cancer specific gene, BCSG1, which was (a) highly expressed in mammary gland relative to other organs and was (b) high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker (1). We demonstrated a stage-specific BCSG1 expression as follows: BCSG1 was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma *in situ*, but was expressed at an extremely high level in advanced infiltrating breast cancer (1). Overexpression of BCSG1 was also demonstrated in ovarian cancer (4).

**A-2. Neural protein synuclein.** Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather, BCSG1 revealed extensive sequence homology to neurotic protein synuclein, having 54% and 56% sequence identity with  $\alpha$  synuclein (SNCA) and  $\beta$  synuclein (SNCB), respectively. Subsequent to the isolation of BCSG1, synuclein  $\gamma$  (5) and persyn (6) were independently cloned from a brain genomic library and a brain cDNA library. In fact, BCSG1, SNCG, and persyn appear to be the same protein. Thus, the previously identified BCSG1, which is also highly expressed in brain (1), has been renamed as SNCG as the third member of synuclein family (7). Synucleins has been specifically implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Mutations in SNCA is genetically linked to several independent familial cases of PD (8). More importantly, wild type of SNCA is the major component of Lewy bodies in sporadic PD (9-10). SNCA peptide known as non-amyloid component of plaques has been implicated in amyloidogenesis in AD (11-12). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy bodies cases (13-14).

**A-3. Expression of BCSG1/SNCG in breast and ovary cancer.** Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, synucleins have also been implicated in non-neural diseases particularly in the hormone responsive cancers of breast and ovary (1,4-5,15-17). Being identified as a breast cancer specific gene, SNCG mRNA was detected in neoplastic breast epithelial cells but not in normal mammary epithelial cells (1). While the expression of SNCG in normal breast is non-detectable (0 out of 7 normal breast specimens), 43% of stage II/III breast carcinomas (6 of 14) and 73% of stage IV breast carcinomas (11 of 15) expressed SNCG, respectively (Fig. 1). Western analysis to examine SNCG protein expression in human breast tissues showed a similar pattern in that it was not detected in normal breast tissues and stage I/II ductal breast carcinomas, but was detected in 70% of Stage III/IV ductal breast carcinomas (12). Ninkina et al were also able to confirm by using Northern and Western blotting that some breast tumors and breast tumor cell lines expressed SNCG, whereas normal breast tissue did not (16). In addition to the link to breast cancer progression, it has also been found that SNCG is involved in ovarian cancer. Following our identification of BCSG1, Lavedan et al first suggested that BCSG1/SNCG may be abnormally expressed in ovarian tumors as well as in breast tumors, based on the discovery of some SNCG ESTs in the libraries derived from an ovarian tumor (5). This suggestion was further confirmed by Western and immunohistochemical analyses (17). While synucleins ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) expression was not detectable by immunohistochemistry in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express either SNCG or SNCB, and 42% (19 of 45) expressed all 3 synucleins ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) simultaneously.

## B. RESEARCH REPORT

**SA1. Does SNCG overexpression in MMTV/SNCG transgenic mice alter mammary gland development?** Finished.

**B-1. Induced highly proliferative pregnancy-like phenotype.** While the transgenic model is still in the process of full characterization, we have so far observed the most robust change in the induction of highly proliferative capability of cells in the terminal end buds. Whole mount preparations of the mammary glands starting at 1-month to 5-month from virgin wild type and virgin transgenic mice were examined to determine

the effect of SNCG on mammary gland development. The homozygous SNCG-transgenic mice were generated. SNCG gene expression in mammary glands was confirmed by RT-PCR and Western blotting. A significant alteration in the developmental pattern of ductal branching was observed in SNCG-transgenic mice compared with the control littermate. While there was limited budding in the wild-type gland (Fig. 1A and C), transgenic glands exhibited multiplicity of ductal branching (Fig. 1B and D). This phenotype was even more dramatic in 5-month old virgin transgenic mice (Fig. 1D). Histological analysis confirmed the presence of multiple ductal branching in mammary glands of transgenic mice (Fig. 2B-D), while there was much fewer ducts in the mammary glands of control mice (Fig. 2A).

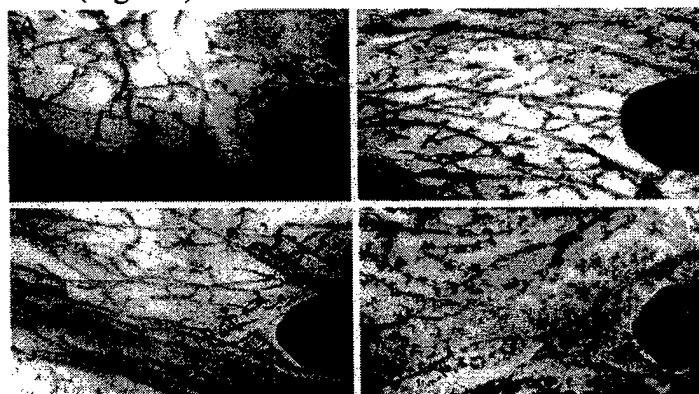


Fig. 1. Whole mount histological analysis of mammary gland from transgenic mouse and wild-type littermate. three-month old (A-B) and 5-month old (C-D) virgin transgenic mice and an age-matched virgin wild-type littermate mice were sacrificed, the right inguinal gland was removed and subjected to whole mount gland fix, defat, and staining. A and C, wild-type control mice. B and D, transgenic mouse. An arrow indicates inguinal lymph node.

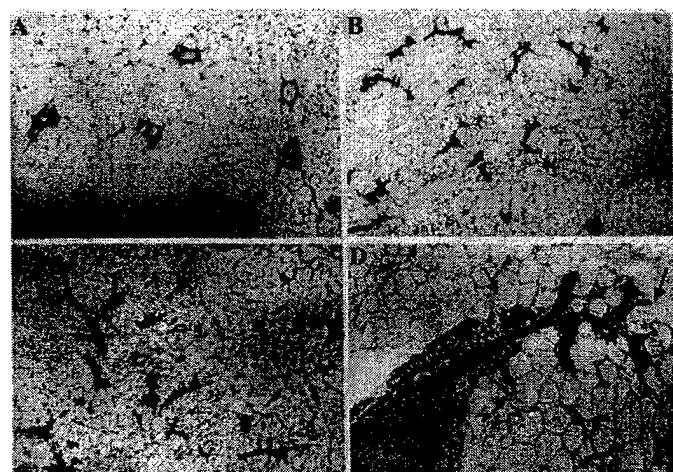


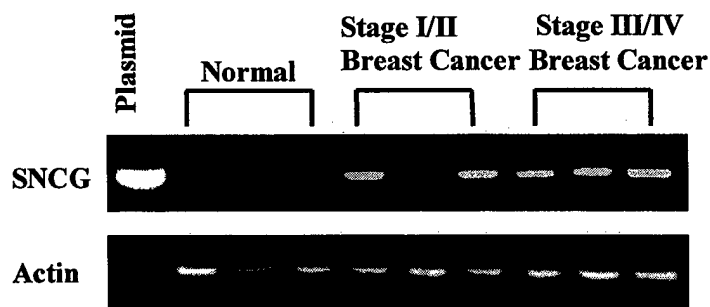
Fig. 2. Histological analysis of mammary gland. Whole inguinal glands were isolated from 3-month (A & B) and 5-month (C & D) virgin mice. A, control mouse. B-D, transgenic mice. A-C, 10 x 10. D, 10 x 40. The proliferation of the cells in end bud or multiple budding was clearly present in the transgenic gland with a significant increase from the gland in 3-month (B) to the gland in 5-month mouse (C). A higher magnification (D) shows a very organized end bud proliferation and branching (arrows indicated).

## SA2. Does overexpression of SNCG in the mammary glands of transgenic mice enhance breast cancer progression?

We already generated the SNCG-transgenic mice. We also generated SNCG and erbB2/Neu transgenic mice by cross-mating the SNCG transgenic mice and erbB2/Neu transgenic mice. We are in the process to determine if SNCG overexpression will stimulate breast cancer incidence and progression induced by DMBA and enhance breast cancer incidence and progression induced by erbB2/Neu overexpression. Because it will take relatively longer time to induce carcinogenesis, we are still awaiting these results.

**B-2. Specific expression of SNCG in advanced breast cancer (20).** Previously, our in situ hybridization analysis has demonstrated an association between SNCG expression and breast cancer progression (1). To further evaluate the clinical relevance of SNCG in a large set of clinical specimens, we performed a semi-quantitative RT-PCR analysis on 79 clinical breast specimens including 12 normal or benign lesions, 2 DCIS, 41 stage I/II breast cancers, and 24 stage III/IV breast

cancers. As shown in **Fig. 3** and summarized in **Table 1**, while no SNCG mRNA was detectable in 12 benign breast specimens, SNCG was expressed in most of advanced infiltrating breast cancers. The expression of SNCG mRNA was detectable in 26 of 67 breast cancer cases (38.8%). Among the 26 of SNCG positive cases, 19 cases were detected in the cancer with stage III/IV, only 6 cases were detected in the cancer with stage I/II, one case was detected in DCIS. There was no detectable SNCG mRNA in 3 cases with tumor size < 2 cm. Therefore, SNCG expression is a stage-specific for breast cancer with no detectable expression in benign lesion, 15% (6/41) expression in stage I/II breast carcinomas, and 79% (19/24) expression in stage III/IV breast carcinomas.



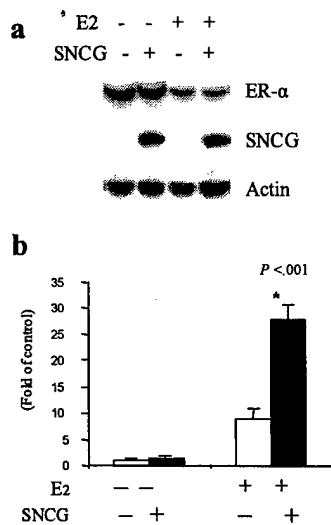
**Fig. 3.** Expression of SNCG in human breast tissue. Total RNA was isolated from frozen human breast specimens. RT-PCR analysis of SNCG expression was conducted using primers within SNCG coding sequence as described in Materials and Methods. The 384-bp PCR product is a specific indication of the presence of SNCG. The integrity and the loading control of the RNA samples were ascertained by actin expression

with a set of primers for 314-bp  $\beta$ -actin. A representative RT-PCR analysis was shown here with 3 benign breast lesions, 3 stage I/II breast cancer cases, and 3 stage III/IV breast cancer cases. The SNCG cDNA plasmid was used as a positive control.

Stage	Benign (n=12)	Stage I/II (n=41)	Stage III/IV (n=24)
Expression	0 (0%)	6 (15%)	19 (79%)

**Table 1.** Stage-specific expression of SNCG on breast cancer. Expression of SNCG in total 79 clinical breast specimens were investigated by 4 RT-PCR analyses. For each round of reaction, we used SNCG c-DNA plasmid as a template for the positive control. The integrity and the loading control of the RNA samples were ascertained by actin expression. Negative cases were confirmed with at least two independent experiments. These patients were randomly selected with the mean age of 58.2 years old, including 67 breast cancers, 7 breast hyperplasias, and 5 fibroadenomas.

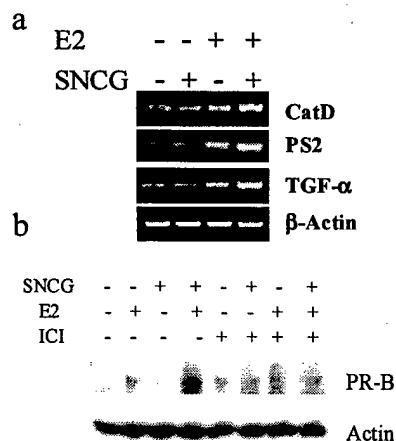
**B-3. Overexpression of SNCG stimulated transcriptional activity of ER- $\alpha$  (21).** Estrogen response is mediated by two closely related members of the nuclear receptor family of transcription factors, ER- $\alpha$  and ER- $\beta$  (18-19). Since ER- $\alpha$  is the major estrogen receptor in mammary epithelia, we measured the effect of SNCG on modulating the transcriptional activity of ER- $\alpha$  in human breast cancer cells. We first selected ER $\alpha$ -positive and SNCG-negative MCF-7 cells as recipient for SNCG transfection (Fig. 4A & B). MCF-7 cells were transiently transfected with either the pCI-SNCG expression plasmid or control pCI-neo plasmid. Transfection of SNCG gene into the SNCG-negative MCF-7 cells did not affect ER- $\alpha$  expression under the conditions both with and without E2 (Fig. 4A). In the absence of E2, the basal levels of ER- $\alpha$  on control and SNCG transfected cells are same. Although treatment of the control cells with E2 resulted in a significant decrease in ER- $\alpha$  level, overexpression of SNCG did not affect E2-mediated degradation of ER- $\alpha$ . Transfection of SNCG significantly stimulated E2-mediated activation of ER- $\alpha$  (Fig. 4B). Treatment of wild-type and SNCG transfected MCF-7 cells with E2 resulted in a significantly differential increase in estrogen-responsive reporter ERE4-Luciferase (ERE4-Luc) activity relative to basal levels in untreated cells. Overexpression of SNCG gene in MCF-7 cells increased E2-stimulated reporter activity 3.2-fold over the SNCG-negative control cells. The SNCG-stimulated transcriptional activity of ER- $\alpha$  was ligand-dependent, because SNCG had no significant effect on the transcriptional activity of ER- $\alpha$  in the absence of E2.



**Fig. 4.** SNCG stimulated ER- $\alpha$  transcriptional activity in MCF-7 human breast cancer cells. Cells were first transiently transfected with pCI-SNCG or the control vector pCI-neo. The transfected cells were selected with G418 and then transfected with pERE4-Luc as well as control reporter pRL-SV40-Luc. After transfection, cells were cultured in the ligand-free medium for 4 days as described in the Conditioned Cell Culture of Experimental Procedures, treated with or without 1 nM E2 for 24 hours before the promoter activities were determined by measuring the dual luciferase activity (A). Western analysis of ER- $\alpha$  and SNCG in MCF-7 cells transfected with pCI-SNCG or the control vector pCI-neo. Expression of SNCG did not affect the ER- $\alpha$  expression in the conditions both with and without 24-h E2 treatment. SNCG stimulated ER- $\alpha$  signaling in MCF-7 cells (B). The ERE reporter luciferase activity was normalized against the control renilla luciferase activity to correct for transfection efficiency. All values were presented as the fold induction over the control luciferase activity in the non-treated SNCG-negative cells, which

was taken as 1. The numbers represent means  $\pm$  SD of three cultures.

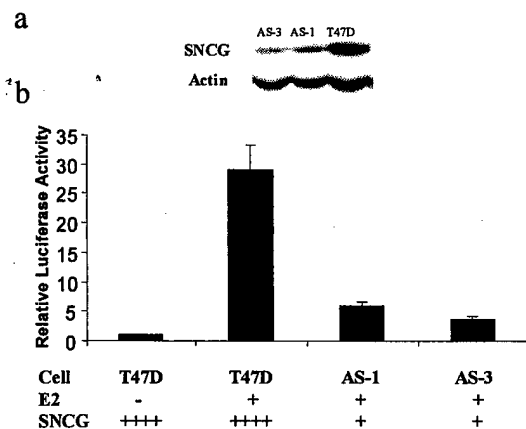
Consistent with the increased transcriptional activity of ER- $\alpha$ , SNCG also stimulated E2-regulated genes in MCF-7 cells (Fig. 5). While SNCG had no effect on the transcription of Cathepsin D (Cat-D), PS2, and TGF- $\alpha$  in the absence of E2, transcription of Cat-D, PS2, and TGF- $\alpha$  were increased 3.9-fold, 3.2-fold, and 4.2-fold in SNCG transfected cells vs. control cells in the presence of E2, respectively (Fig. 5A). To evaluate the effect of anti-estrogen on SNCG-stimulated ER- $\alpha$ -regulated genes, we treated the cells with an antiestrogen ICI 182,780 (ICI). As demonstrated in Fig. 5B, the basal levels of PR were very weak in both SNCG-transfected and control cells, but were increased significantly by E2 treatment. Treatment of the cells with E2 stimulated a 3.5-fold PR protein expression in SNCG transfected cells compared with control cells. Although ICI slightly stimulated basal levels of PR, treatment of the SNCG-transfected MCF-7 cells with ICI significantly blocked E2-stimulated PR expression, indicating that SNCG-stimulated gene expression in E2 treated cells is mediated by ER- $\alpha$ .



**Fig. 5.** SNCG stimulated estrogen-regulated gene transcription in MCF-7 cells. Cells were transiently transfected with pCI-SNCG or the control vector pCI-neo. After G418 selection, cells were cultured in the ligand-free medium for 4 days as described in the Conditioned Cell Culture of Experimental Procedures. A. RT-PCR. Cells were treated with or without 1 nM of E2 for 8 hours before the isolation of total RNA. Expressions of mRNA of Cat-D, PS2, and TGF- $\alpha$  were studied in SNCG transiently transfected cells vs. control cells by RT-PCR analyses. A an 842-bp product of Cat-D, a 336-bp product of PS2, and a 240-bp product of TGF- $\alpha$ , were amplified by RT-PCR and normalized with actin. B. Inhibition of SNCG-stimulated PR protein expression by antiestrogen ICI. Cells were treated with or without 1 nM of E2 and 1  $\mu$ M of ICI for 32 h. Total proteins were isolated, normalized, and

subjected to Western analysis using anti-PR antibody.

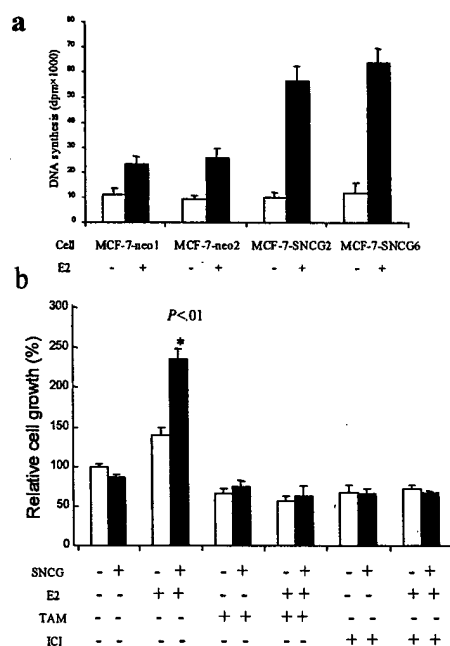
**B-4. Reduced levels of SNCG compromised transcriptional activity of ER- $\alpha$ .** The effect of SNCG expression on ER- $\alpha$  transactivation was further demonstrated by inhibiting endogenous SNCG expression with SNCG antisense mRNA in T47D cells that express high levels of SNCG (8). Stable transfection of the SNCG antisense construct into T47D cells significantly reduced SNCG expression to 25% of that in control T47D cells (Fig. 6A). While E2 significantly stimulated the reporter activity in the control T47D cells, inhibition of SNCG expression reduced E2-responsive luciferase activity in two stable antisense-transfected T47D cell lines, AS-1 and AS-3 cells, to 21% and 13% of that in control T47D cells, respectively (Fig. 6B). Treatment of T47D cells



with E2 resulted in a 25-fold increase over the non-treated cells. However, only 3.3- and 5.2-fold increase was observed in the AS-3 and AS-1 cells, respectively. Taken together, the increased estrogen-responsive reporter activity in SNCG transfected MCF-7 and MDA-MB-435 cells as well as the increased estrogen-regulated gene transcription and the compromised transcriptional activity of ER- $\alpha$  in SNCG antisense transfected T47D cells indicated that SNCG stimulated ligand-dependent transcriptional activity of ER- $\alpha$ .

**Fig. 6.** Inhibition of SNCG expression reduced the transcriptional activity of ER- $\alpha$ . (A). Western analysis of SNCG expression in control T47D and SNCG antisense transfected AS-1 and AS-3 cells. (B). ERE-Luc reporter activity in control and antisense transfected T47D cells. Cells were cultured in the ligand-free conditioned medium for 4 days, treated with or without  $10^{-11}$  M of E2 for 24 h before harvesting. All values were normalized to the reporter activity of the non-treated T47D cells, which was set to 1. The numbers represent means  $\pm$  SD of three cultures.

**B-5. Stimulation of cell proliferation by SNCG (22).** To determine the biological relevance of SNCG-stimulated ligand-dependent ER- $\alpha$  signaling, we analyzed the effect of SNCG overexpression on the growth of breast cancer cells. To determine whether SNCG overexpression affects ligand-dependent or ligand-independent cell growth, the cellular proliferation of the previously established two stable SNCG-transfected MCF-7 cell clones, MCF-SNCG2 and MCF-SNCG6, were compared with that of SNCG-negative cells, MCF-neo1 and MCF-neo2 (16). Data in Fig 7A shows that while SNCG had no significant effect on the proliferation of SNCG-transfected cells compared to MCF-neo cells in the absence of E2, overexpression of SNCG significantly stimulated the ligand-dependent proliferation. Treatment of neo clones with E2 stimulated an average cell proliferation 2.4-fold over controls. However, E2 treatment of SNCG clones resulted in an average of 5.4-fold increase in the proliferation vs. controls, suggesting that SNCG expression renders the cells more responsive to E2-stimulated cell proliferation. To address whether the stimulatory effect of SNCG on cell growth is mediated by ER- $\alpha$ , we investigated the effect of the antiestrogen tamoxifen and ICI. As shown in Fig. 7B, E2-stimulated growth in both MCF-neo1 and SNCG-MCF6 cells was significantly blocked by tamoxifen and ICI. These data indicate that SNCG-stimulated cell growth is mediated by ER- $\alpha$ .



**Fig. 7.** SNCG stimulated ligand-dependent cell proliferation. For all experiments, cells were cultured and synchronized in the ligand-free Conditioned Cell Culture medium for 4 days before the hormone treatments. A, Stimulation of cell proliferation by SNCG overexpression. Cells were treated with or without 1 nM E2 for 24 hours. Cell proliferation was measured by  $^3$ H- thymidine incorporation. Data are means  $\pm$  SD of three cultures. B, Effect of antiestrogens on SNCG-stimulated cell growth. Cells were treated with or without 1 nM of E2, 1  $\mu$ M of tamoxifen, or 1  $\mu$ M of ICI for 6 days before harvesting. Media were changed every two days with fresh estrogen and antiestrogens. Cell growth was measured using a cell proliferation kit (XTT). Data are the mean  $\pm$  SD of quadruplicate cultures. Open bar represents MCF-neo1 cells; closed bar represents MCF-SNCG6 cells.



**B-6. SNCG enhances ligand-binding by estrogen receptor and functions as a HSP70 cochaperone (22).** To determine the mechanisms underlying SNCG's stimulation of estrogen receptor signaling, we found that SNCG is a novel Hsp70/Hsc70 cochaperone. As a Hsp70 binding protein, SNCG participated in Hsp70-Hsp90-ER multiproteins complex. The high-affinity ligand-binding by ER was significantly enhanced by SNCG overexpression. We also found that SNCG significantly stimulated ligand-dependent mammary tumorigenesis (22).

### **C. KEY RESEARCH ACCOMPLISHMENTS**

For the first time, we found that:

**SNCG overexpression in mammary glands induces a proliferative phenotype and alter mammary gland development.**

**SNCG stimulates ligand-dependent estrogen receptor signaling**

**SNCG is a novel Hsp70/Hsc70 cochaperone**

**SNCG enhances ligand-binding by estrogen receptor**

**SNCG stimulates ligand-dependent mammary tumorigenesis**

Two research articles were published:

**Yangfu Jiang, Ellie Y. Liu, Aiping Lu, et al.** Stimulation of estrogen receptor signaling by gamma synuclein. *Cancer Research*, 63 (14): 3899-3903, 2003

**Yangfu Jiang, Ellie Y. Liu, Ithzak D. Goldberg, Y.Eric Shi.** Gamma synuclein, a novel heat-shock protein-associated chaperone, stimulates ligand-dependent estrogen receptor signaling and mammary tumorigenesis. *Cancer Research*, 64: 4539-4546, 2004

### **D. CONCLUSIONS**

1. Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, they have also been implicated in non-neural diseases, such as breast cancer and ovarian carcinoma. SNCG expression is highly associated with breast cancer progression. Overexpression of SNCG in breast cancer cells stimulated cell growth and tumor metastasis. We also found that SNCG stimulates the ligand-dependent estrogen receptor signaling mammary tumorigenesis.
2. Here, we also reported that SNCG is a novel Hsp70-associated protein. By functioning as a Hsp70 cochaperone, SNCG enhances the high-affinity ligand-binding by estrogen receptor, thereby stimulating estrogen receptor signaling.
3. In addition, we also found that SNCG expression in the mammary glands of transgenic mice significantly promoted a proliferative phenotype and resulted in multiple ductal branching.

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